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Physical approaches to the study of chromatin fibers

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Summary

Investigations of the structures of complex macromolecular assemblies (like chromatin fibers, microtubules, etc.) have traditionally utilized two approaches, which we term *macroscopic* and *microscopic*. The macroscopic methods include hydrodynamic and radiation-scattering techniques. While applicable to molecules in solution, they present results which are only averages over the molecules in the sample. If, as is usually the case, these structures are heterogeneous, interpretation can become hopelessly ambiguous. At the other extreme, the traditional high-resolution microscopic techniques (transmission electron microscopy and its numerous variants) while sensitive to even local variations in structure, impose often devastatingly harsh conditions on delicate biological structures. Recently, two kinds of microscopic methods have been developed which hold great promise for studies of macromolecular assemblies. The first is cryo-electron microscopy, which allows preservation of much of solution structures. Second, and potentially even more promising, are the various scanning probe microscopic methods, especially scanning force microscopy. In its present stage of development, this technique allows detailed structural studies under relatively mild conditions. Together, cryo-electron microscopy and scanning force microscopy have already provided new insights into the static structure of chromatin. Even more exciting are the prospects for imaging in liquid media, now under development. These hold promise for study of not only the statics, but dynamics as well, for functionally important structures like chromatin fibers.

I. Introduction

Elucidation of the fine structures of complex, irregular, and asymmetric macromolecular assemblies has always presented an especially difficult problem for molecular biologists. As molecular biology ascends toward the cellular level, the fact that structures which exhibit neither regular periodicity nor symmetry clearly constitute a major class of higher order subcellular organization makes the problem of increasing importance. An example which has attracted much attention in recent years is the interphase chromatin fiber. Despite efforts to impose regularity in terms of various kinds of model fiber folding, it is clear even from studies of composition that the fiber must be heterogeneous along its length. It also must be subjected to major perturbations in vivo as processes like transcription and replication occur.

The problems presented by heterogeneity are further complicated by the fact that we wish to study objects like the chromatin fiber in an environment as close as possible to the physiological one. This means, at a minimum, that certain ionic conditions should be maintained in the surrounding medium; in actuality the proper conditions for study may be much more complex than this, often requiring the presence of ongoing metabolic processes.

Two general approaches have been taken toward the physical study of such structures. In one class of techniques, which we shall call macroscopic, a solution of the macromolecular structures, in a defined solvent medium, is examined by one or another of various hydrodynamic methods or by the scattering of radiation (light, X-rays, neutrons). These techniques have the advantage that the medium can be made to at least approximate the in vivo environment, and can be varied at will. On the other hand, these methods suffer from the major disadvantage that only average properties of a highly heterogeneous molecular population are observed.

In the second class of techniques, which we call microscopic, individual macromolecular complexes are observed by one or another of several microscopic

methods capable of resolution to a few nanometers or better. This allows, in principle, the observation of local variations in the fiber, or perturbations in its structure, without the disadvantage of averaging. Until very recently, however, all microscopic methods capable of the requisite resolution required conditions of fixation, staining, and/or dehydration wholly incompatible with the native environment of macromolecular assemblies.

It is the purpose of this review to briefly describe the advantages and limitations of each kind of technique, and to attempt to point out the new directions which methodology is taking in attempts to circumvent the restrictions described above. Although we will concentrate, for an example, on the chromatin fiber, much of what will be described is applicable to any of the giant, irregular macromolecular assemblies which form the functional components of cells.

II. Macroscopic techniques

The classical methods for the study of the structures of macromolecular assemblies in solution can be divided into hydrodynamic and scattering methods. These are all old techniques; the basic principles were elucidated in the period between 1920 and 1940; the subsequent advances have been mainly in applications and instrumentation.

A. Hydrodynamic studies of chromatin fibers.

Sedimentation and diffusion measurements have found their principal application to study of chromatin fibers by virtue of the fact that both the sedimentation coefficient (S) and the diffusion coefficient (D) are related to the frictional coefficient (f). That is

$$S = M(1-\bar{v}\rho)/Nf \quad (1)$$

$$D = RT/Nf \quad (2)$$

where M denotes molecular mass, $\bar{v}$ partial specific volume, ρ solution density, N Avogadro's number, R the gas constant and T the absolute temperature. The frictional coefficient is a measure of the resistance offered by the surrounding solution to motion of a macromolecule. It depends upon the size and shape of the particle, but in a manner so complex that it is difficult or impossible to extract quantitative structural information on complex structures from a measure of f. However, changes in the frictional coefficient can be readily measured and can often be interpreted, in at least a semi-quantitative way, in terms of changes in fiber structure. To take one example: the extensive studies of Thomas and coworkers (Butler and Thomas, 1980; Thomas and Butler, 1980), using carefully purified chromatin fractions, exhibit clear evidence for condensation and expansion of chromatin fibers in response to changes in the ionic environment. If sedimentation measurements are to be interpreted in terms of f, one must ascertain in some manner that association of fibers (which would change M) is not involved.

Often, such methods are most powerful when applied to simplified systems. An example is found in the study of "chromatin" formed by reconstitution of histone octamers onto dodecameric repeats of a sea urchin 5S gene. Each repeating unit of the construct forms, upon addition of histone octamers, a quite accurately positioned nucleosome, so that the dodecameric repeat contains twelve nearly equally spaced nucleosomes. With such reconstitutes, one has both homogeneity and near-regularity of structure, and it has been possible in such cases to predict the frictional coefficient (or sedimentation coefficient) expected for various possible foldings of the fiber (Hansen et al., 1989). Because the molecules are homogeneous in size, it was easy to eliminate the possibility of aggregation by sedimentation equilibrium studies. Unfortunately, even in such idealized cases a residuum of ambiguity remains, for there exists a range of different structures which can provide the same value of the sedimentation coefficient (**Figure 1**). Thus, no unique solution with respect to structure is provided.

Figure 1. Two different models for a dodecameric oligonucleosome that predict almost exactly the same sedimentation coefficient. In Panel A is shown one possible conformation of "beads-on-a-string" model, in which the absence of linker histones has led to partial unwrapping of DNA from histone cores, and a random-coil arrangement of nucleosomes. In Panel B is

a planar zig-zag model, with fixed linker lengths, 1.75 turns of DNA per core particle, and 90˚ linker-linker angles. Both, using the Kirkwood theory for frictional coefficients (van Holde, 1985), predict about 29S for the sedimentation coefficient of a dodecamer of 11S subunits.

The major disadvantage of such methods, obviously, is that a single average quantity is measured, and variations in that quantity can be interpreted in multiple ways. Only in the very simplest systems does interpretation become unambiguous. Thus, for example, the fact that the sedimentation coefficient of dinucleosomes does not change with salt concentration under conditions where the value for trinucleosomes does argues strongly against linker bending as salt increases (Butler and Thomas, 1980; van Holde and Zlatanova, 1996). In a similar vein we note that although early hydrodynamic studies of chromatin fibers did not suggest nucleosomal structure, such experiments were very important in the initial characterization of the nucleosomes themselves (Sahasrabuddhe and van Holde, 1974).

Other hydrodynamic methods, which involve rotational diffusion of macromolecules (electric dichroism and electric birefringence, for example) have been employed infrequently in chromatin studies (see van Holde, 1988, for details). The measurement of rotational diffusion is exceedingly sensitive to changes in molecular asymmetry, which is an advantage (van Holde, 1985). However, it is also very sensitive to heterogeneity, and chromatin fiber fragments are rarely of even approximately uniform size.

It is our opinion that the era in which population-averaging hydrodynamic methods provided important information about macromolecular structures is drawing to a close, except for application to very special problems. Too little information is gained from time-consuming experiments that require, in most cases, relatively large quantities of sample.

B. Scattering methods.

Studies of the scattering of various kinds of radiation (light, X-rays, neutrons) from solution can provide information concerning the dimensions, and in some cases, internal structure of dissolved macromolecular assemblies. This is basically because the way in which scattered intensity varies with angle reflects constructive and destructive interference between photons scattered from various parts of the structure (**Figure 2**). The shorter the wavelength, the finer the detail that can be probed.

The use of the scattering of radiation to investigate chromatin fiber structure has a long history. Indeed, soon after the remarkable successes obtained with X-ray scattering from DNA fibers, attempts were made to carry out similar studies with fibers of chromatin (see, for example, Wilkins et al., 1959; Pardon et al., 1967). However, it has proved difficult to obtain well-oriented fibers of chromatin, so others turned to low-angle X-ray scattering of unoriented samples in solution (see Bram and Ris, 1971, for an early example). None of these early scattering studies was interpreted in a manner to suggest the kind of repeating nucleosomal structure soon to be recognized by other techniques. This illustrates a common failing of scattering methods; except for extremely regular structures (such as crystals) scattering measurements do not define structures. Rather, like hydrodynamic methods,

Figure 2. The kinds of information that can be obtained from low-angle scattering. One way to treat the data is shown here, where the log of scattering intensity at angle (θ) is plotted versus $\sin^2\theta$. The intercept (A) at θ=0 gives the mass of the scattering particles, the initial slope (B) gives the radius of gyration, and the pattern of maxima and minima at higher angles is sensitive to internal structure of the particles. For long, rod-like particles like chromatin fibers, a different analysis of the same kind of data can yield the mass per unit length and cross-section radius of gyration.

they can provide measures of specific average quantities (mass per unit length, cross-section radius of gyration, etc.). The much less intense scattering observed at higher angles provides the possibility of extracting further information (see **Figure 2**), but even so, the problem usually reduces to one of fitting one or another model to the scattering curve. Finally, unless samples can be highly oriented (and chromatin fibers have been notoriously difficult to orient) all measurements involve an averaging over rotationally random particles, which necessarily loses information.

Nevertheless, once the nucleosomal repeating structure of chromatin was deduced, a variety of scattering methods were quickly applied to provide further information about the nucleosomes and the polynucleosomal fiber. These included neutron scattering studies of the nucleosomal core particle (Pardon et al., 1975; Suau et al., 1977) which provided the first physical evidence that the DNA was coiled outside a histone core, in addition to light scattering (Campbell et al., 1978), low angle X-ray (Finch and Klug, 1976) and neutron scattering (Suau et al., 1979) studies of the fibers. The fiber studies were particularly useful in corroborating the early evidence on chromatin fiber structure from electron microscopy, demonstrating that the dimensions such as the average cross-section radius of gyration and the average mass per unit length observed in aqueous solution were at least broadly commensurate with those observed under the extreme conditions of dehydration required for electron microscopy (see below).

The possibility of using contrast-variation (see van Holde, 1985), gives neutron scattering special advantages in the study of chromatin fibers. Because the DNA and protein components of the chromatin fiber have distinctly different neutron scattering power, it has been possible to provide some evidence concerning the distribution of mass in the fibers. Further, the fact that deuterated proteins scatter neutrons differently than proteins containing only ^{1}H has allowed investigation of the (again averaged) position of linker histones within the fiber; to that end, chromatin fibers were depleted of linker histones and then reconstituted with deuterated ones (Graziano et al., 1994).

In comparison with hydrodynamic techniques, the scattering methods are clearly capable of providing more detailed information about fiber structure; the curve of scattering intensity vs angle (**Figure 2**) contains much more information concerning shape and internal structure than does a single hydrodynamic parameter. This is especially true of neutron scattering, although this technique suffers from the limitation that it can be effectively carried out in only a few laboratories. Low-angle X-ray scattering also shows future promise, especially if either better methods for aligning fibers are obtained, or ultra-high intensity sources become available for fast dynamic experiments.

III. Macroscopic vs microscopic techniques

There is an enormous gulf dividing the macroscopic methods we have described above from the microscopic techniques that can look at individual fragments of chromatin. The macroscopic methods yield numbers (sedimentation coefficients, repeat distances, radii of gyration, etc.) which are typically averages over an immense number of individual objects which themselves are locally heterogeneous in structure. Therefore, attempts to use such experiments to answer biological questions are frequently frustrating. What, for example, can the sedimentation coefficient or mean square cross-section radius of gyration tell us about the structure of transcriptionally active chromatin when the sample contains an unknown mix of "active", "potentially active" and "inactive" chromatin? Unless some kind of fractionation is used (and such methods are notoriously inefficient to date) each macroscopic technique averages (in one of several possible ways) over the whole spectrum of fiber structures in the sample. Probably the least rewarding experience in chromatin studies over the past three decades have been the attempts to separate "active" from "inactive" chromatin on a scale allowing meaningful macroscopic examination.

There is one potential way out of this dilemma. It is now possible to reconstitute at least some features of the chromatin structure in vitro. This might allow us, in principle, to make the kinds of fiber structure we believe to be representative of repressed or active chromatin. Unfortunately, we do not know the ground rules. If we want to make something like active chromatin structure in vivo, what are the ingredients? How much histone acetylation do we need? How much linker histone? How much non-histone chromosomal protein, and of what varieties? One can envision an essentially infinite research project, ringing all the possible variations on these themes.

It is a contention of this paper that the future advances in biochemistry and molecular biology are going to come not from macroscopic studies of immense numbers of molecules, but from microscopic studies conducted in solvent media, frequently at the single-molecule level. The events that occur in processes like transcription and replication are simply too delicate to be studied with the blunt tools of classical physical biochemistry. We shall be examining, more and more, the interaction of one enzyme with one substrate molecule, the movement of one kinesin molecule along one microtubule, the passage of one polymerase along one DNA sequence. For such measurements to be meaningful, to be assured that we are not simply observing one aberrant event in the molecular chaos, we must substitute repetition in time for repetition in numbers. Consequently, we predict that a forthcoming advance in "single-molecule-chemistry" will be the

development of various kinds of automation of experiments to allow repetitive observation of a given process.

IV. Microscopic methods

In an attempt to avoid the informational limitation inherent in macroscopic methods, researchers have turned more and more toward microscopic methods. Indeed, the possibility to examine in detail the structures of long chromatin fibers provides the possibility to seek out local heterogeneities which can at least delineate the limits of fiber structures and may be clues to function. However, the microscopic techniques have traditionally been beset by other problems equally serious to those facing macroscopic methods. We can best describe these by first presenting a brief overview of the applications of electron microscopy to the study of chromatin fibers. We shall then, in the final section, describe some new microscopic techniques, and how we see their potential for chromatin fiber analysis.

A. Electron microscopy (EM) of chromatin fibers.

The first useful studies of structures like the chromatin fiber at the nanometer level utilized transmission electron microscopy. Indeed, it was the application of the spreading technique of Miller and Beatty (1969) that allowed Olins and Olins (1973,1974) and Woodcock (1973) to obtain the first indications of a repeating structure in chromatin. Over the subsequent years, many careful and sophisticated studies have been carried out by this method, and many of our current ideas about the fiber structure are based upon these (see, for examples Finch and Klug, 1976; Thoma et al., 1979; Woodcock et al., 1984; Williams et al., 1986). Nonetheless, conventional transmission EM requires such serious abuse of the sample that practitioners have been concerned from the very first. Samples must in most cases be fixed, stained or shadowed, and then subjected to dehydration in a hard vacuum. To what extent are observed structural features artifacts of such treatment?

In the past decade, a new EM technique has emerged which goes a long way toward circumventing these difficulties. This is cryo-EM, in which samples are quickly frozen in a film of vitreous ice and then examined by transmission EM (see Dubochet et al., 1992, for a review). Fixation is unnecessary, and image enhancement techniques can circumvent staining. If sublimation can be limited, the fiber is in essentially the solution (albeit frozen) in which it was prepared, and no substantial dehydration should have occurred. Studies of chromatin fibers (Bednar et al., 1995; Woodcock and Horowitz, 1995) by this technique exhibit nucleosomes in irregular, helix-like structures, which bear only limited resemblances to the picture that has been derived from conventional EM.

Although cryo-EM represents, in our opinion, a major step toward the unbiased examination of supramolecular organization, it still suffers from some potential problems and major limitations. It is difficult to be certain that the process of freezing to very low temperatures, rapid as it may be, does not induce some changes in the structure. Furthermore, the necessary thinness of the ice film (~100 nm) creates the possibility that larger macromolecular fibers be distorted by this confinement (see Dubochet et al., 1992, for discussion). A limitation lies in the fact that the sample is frozen; therefore, any direct investigations of the dynamics of macromolecular function are precluded, although fast freezing can provide serial sampling.

B. Scanning probe microscopy.

In the past 15 years, a group of entirely new techniques have emerged which show promise of revolutionizing our approach to many biological problems. There are a number of techniques going under the general term scanning probe microscopy; all share the characteristic that a finely pointed probe traverses the sample, and senses, in one way or another, surface features. A compendium of recent references to all of these methods is provided by Bottomley et al. (1996). Some of these techniques are probably unsuitable for biological studies but two have had considerable impact—scanning tunneling microscopy and scanning force microscopy—SFM (also called atomic force microscopy—AFM). This latter method has found the widest application in biology to date, and will be discussed here. In SFM a probe, with tip curvature of the order of 10 nm or less, is mounted atthe end of a very flexible cantilever arm. Deflections of the arm as the tip traverses the sample are amplified by an optical lever (**Figure 3**). The sample is usually deposited on atomically flat mica or glass surface.

Figure 3. Schematic drawing of a scanning force microscope. In the tapping

mode the cantilevered tip is caused to oscillate, and the sample is scanned by piezoelectric deflection of the sample stage. The tip oscillations are detected by an optical lever, a laser beam reflected off the cantilever onto a split photodiode. A feedback circuit to the piezoelectric crystal raises or lowers the sample stage so as to keep the amplitude of oscillation constant. This signal measures the height at each point in the sample.

Figure 4. SFM image of unfixed chicken erythrocyte chromatin fibers on glass substrate, deposited from low ionic strength (5 mM triethanolamine) buffer. Individual nucleosomes can be resolved. The height of each nucleosome above the surface is indicated by the shading; highest ones are lightest. Even at this low ionic strength, the fiber is not fully extended or flattened, but forms an irregular, helix-like structure.

The scanning force microscope can operate in a number of modes. In the contact mode, the probe tip is simply drawn across the surface. In the tapping mode, the tip is made to oscillate, and thus "taps" its way across the surface of the sample. In general, the tapping mode is found to produce less distortion to biological samples (see Bustamante and Keller, 1995; Shao and Yang, 1995). With currently available instrumentation, resolution of a few nanometers is readily available.

This technique has been employed by us (Leuba et al., 1994; Yang et al., 1994) and by others (Allen et al., 1993) to the study of chromatin fiber structure. The advantages over conventional transmission EM are several: staining is never required, and in some cases even fixation can be dispensed with (see **Figure 4**). Samples can be studied "in air", or in some cases under liquid. Even the "in air" samples, though dried at moderate relative humidity, are not desiccated as in the high vacuum of the EM, and retain water of hydration. At low ionic strength, individual nucleosomes in the fiber are easily resolved and the image shows, in terms of shading, the relative height of each nucleosome above the surface. Thus, the three-dimensional coordinates of each nucleosome can be

measured, and distribution of nucleosome-nucleosome distances, internucleosome angles and fiber heights easily accumulated (**Figure 5**).

The investigations reveal a somewhat different picture of the chromatin fiber at low ionic strength than had been envisioned from conventional EM studies—although one more commensurate with some earlier scattering experiments. At low salt, the fibers are rather open, irregular helix-like structures, a kind of conformation which can be predicted from simple assumptions about linker DNA behavior (see Leuba et al., 1994; Yang et al., 1994; Woodcock and Horowitz, 1995; van Holde and Zlatanova, 1996). In further studies, it has been possible to exploit the ease of quantitation of such digitized images to explore the effects on structure of proteolytic removal of portions of histone molecule from the chromatin fiber (Leuba et al., submitted). There remain serious limitations to the kind of studies described above. To date, we have not been able to resolve nucleosomes in the more closely packed condensed fibers formed at higher ionic strength. Most important is the fact that air drying, although avoiding the ravages of total dehydration, may still represent a static, non-native condition.

For this reason, there is currently enormous interest in the newest development in this technique—the possibility of operating tapping mode SFM under liquid. If this can be accomplished with resolution comparable to that obtained by tapping mode in air, a whole new world of experimentation will be opened up. It should then be possible to observe directly the changes in chromatin structure accompanying changes in protein composition or ionic medium, or covalent modification of the histones themselves. Direct, real-time observation of the interaction of enzymes and transcription factors with the chromatin fiber should also be possible.

Figure 5. Quantitative data on chicken chromatin fiber structure from SFM experiments. By measuring several thousand nucleosomes, in a number of fibers, both average values and distributions of (A) center-center distances, (B) internucleosome angles, and (C) heights of nucleosomes above substrate could be determined.

There has already been considerable success in studying DNA, and some DNA-protein interactions in buffer. For example, Lyubchenko and Shlyakhtenko (1997) have studied the conformational changes of supercoiled DNA in response to different ionic strengths in just this way. The dynamic interaction of E. coli RNA polymerase with DNA has been demonstrated, under buffer, by Kasas et al. (1997). It seems likely that these techniques can be extended, in the very near future, to the investigation of the structure and dynamics of the chromatin fiber.

The major problems remaining may be largely concerned with how to appropriately attach the fibers to the surface to be studied. Too weak a fixation will result in release, or at least excessive Brownian motion of fiber segments during observation, leading to loss of resolution. On the other hand, too firm a fixation may prevent the required conformational changes in response to alterations

in the environment or interaction with other molecules. These seem, however, to be technical problems of the sort that are ultimately resolved by skilled experimenters. When this is done, the investigation of dynamic processes in chromatin will at last be possible.

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